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(11) EP 0 922 759 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication: 16.06.1999 Bulletin 1999/24  
(51) Int. Cl.<sup>6</sup>: C12N 9/02, C12P 7/60  
// (C12N9/02, C12R1:01)  
(21) Application number: 9812223.5  
(22) Date of filing: 23.11.1998

(84) Designated Contracting States:  
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE  
Designated Extension States:  
AL LT LV MK RO SI  
(30) Priority: 01.12.1997 EP 97121089  
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(54) Aldehyde dehydrogenase

(57) A new aldehyde dehydrogenase having the physico-chemical properties: molecular weight: 150,000 ± 6,000 or 230,000 ± 9,000, substrate specificity: active on aldehyde compounds; cofactors: pyrrolo-quinoline quinone and heme c; optimum pH: 7.0-8.5; and inhibitors: Co<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, monoiodoacetate and EDTA, is derived from a microorganism belonging to the genus *Gluconobacter*. Said aldehyde dehydrogenase can be produced by cultivating a microorganism of the genus *Gluconobacter* which is capable of producing an aldehyde dehydrogenase having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism. 2-Keto-L-gulonic acid (2-KGA) can be produced from L-sorbose by contacting L-sorbose with (i) the aldehyde dehydrogenase in the presence of an electron acceptor, (ii) a *Gluconobacter* microorganism capable of producing the aldehyde dehydrogenase in an aqueous medium under aerobic conditions or (iii) a cell-free extract of said microorganism, and in each case isolating the resulting 2-KGA from the reaction mixture.

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## Description

[0001] The present invention concerns a novel enzyme, namely aldehyde dehydrogenase (ADH), a process for producing ADH and a process for producing 2-keto-L-gulonic acid ( 2-KGA ) from L-sorbose utilizing said enzyme. 2-KGA is an important intermediate for the production of vitamin C.

[0002] Some microorganisms are known to convert L-sorbose to 2-KGA. For example, in U.S. Patent Specification No.3907639, the microorganisms belonging to the genera *Acetobacter*, *Pseudomonas*, *Escherichia*, *Serratia*, *Bacillus*, *Staphylococcus*, *Aerobacter*, *Alcaligenes*, *Penicillium*, *Candida* and *Gluconobacter* are reported to be capable of effecting the conversion. Furthermore, Kitamura et al. (Eur. J. Appl. Microbiol., 2, 1, 1975 ) report that the enzyme oxidizing L-sorbose found in *Gluconobacter melanogenus* IFO 3293 requires neither a coenzyme nor an electron acceptor for the development of enzyme activity. Makover et al. (Biotechnol. Bioeng: 17, 1485, 1975 ) report the presence of L-sorbose dehydrogenase activity in the particulate fraction of *Pseudomonas putida* ATCC 21812 and of *Gluconobacter melanogenus* IFO 3293. They also indicate that neither nicotinamide adenine dinucleotide (NAD) nor nicotinamide adenine dinucleotide phosphate (NADP) acts as a coenzyme for the enzyme. T. Hoshino et al. (Agric. Biol. Chem., 55, 665, 1991) purified and characterized L-sorbose dehydrogenase from *Gluconobacter melanogenus* UV10, which requires NAD or NADP as a coenzyme.

[0003] In the context of the present invention, microorganisms belonging to the genus *Gluconobacter* have been studied more closely and, as a result, it has been found that the further novel ADH which catalyzes the oxidation of L-sorbose to 2-KGA can be obtained from said microorganisms. Furthermore, it has been found that the purified ADH provided by the present invention oxidizes L-sorbose to 2-KGA in the presence of electron acceptors, such as 2,6-dichlorophenolindophenol ( DCIP ) and phenazine methosulfate ( PMS ), ferricyanide or cytochrome c, but that NAD, NADP and oxygen are not suitable as electron acceptors. Thus, the ADH provided by the present invention is clearly distinct from the known L-sorbose dehydrogenase.

[0004] It is an object of the present invention is to provide the novel ADH which acts on L-sorbose to 2-KGA and has the following physico-chemical properties:

a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$  )

b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone (PQQ ) and heme c

d) Optimum pH : 7.0 to 8.5

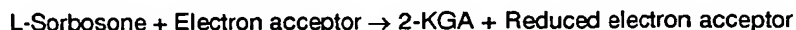
e) Inhibitors :  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid

[0005] It is another object of the present invention is to provide a process for producing the novel ADH of the invention, as defined above, by cultivating a microorganism belonging to the genus *Gluconobacter*, which is capable of producing the ADH having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the ADH from the cell-free extract of the disrupted cells of the microorganism. Yet another object of the present invention is to provide a process for producing 2-KGA from L-sorbose utilizing the ADH of the present invention, which process comprises contacting L-sorbose with (i) the ADH, as defined above, in the presence of an electron acceptor, or (ii) a microorganism belonging to the genus *Gluconobacter* which is capable of producing the ADH, as defined above, in an aqueous nutrient medium under aerobic conditions, or (iii) a cell-free extract of said microorganism, and in each of the cases (i), (ii) and (iii) isolating the resulting 2-KGA from the reaction mixture.

[0006] The physico-chemical properties of the purified sample of the ADH, prepared according to the Examples presented hereinafter, are as follows:

#### 1) Enzyme activity

[0007] The ADH of the present invention catalyzes the oxidation of L-sorbose to 2-KGA in the presence of an electron acceptor according to the following reaction equation:



[0008] The enzyme does not work with oxygen as an electron acceptor. This was affirmed by the failure of the enzyme

to convert L-sorbose to 2-KGA using oxygen as a possible electron acceptor. Furthermore, no oxygen consumption was detected in the reaction mixture as detected with a dissolved oxygen probe. However, any conventional compound which has the ability to act as an electron acceptor can be utilized in conjunction with the enzyme of this invention. DCIP, PMS, ferricyanide and cytochrome c are preferred electron acceptors.

[0009] The enzyme assay was performed as follows:

[0010] The reaction mixture for assaying the ADH activity consisted of 0.1 mM DCIP, 1.0 mM PMS, 50mM potassium phosphate buffer ( pH 8.0 ), 1.0  $\mu$ M PQQ, 2.0 mM L-sorbose and enzyme solution in a final volume of 100  $\mu$ l with water, which reaction mixture was prepared just before the assay. The reaction was started at 25°C with L-sorbose, and the enzyme activity was measured as the initial reduction rate of DCIP at 600 nm. One unit of the enzyme activity was defined as the amount of the enzyme catalyzing the reduction of 1  $\mu$  mole DCIP per minute. The extinction coefficient of DCIP at pH 8.0 was taken as 15 mM<sup>-1</sup>. A reference cuvette contained all the above constituents except L-sorbose.

[0011] The protein concentration was measured with the BCA protein assay reagent ( Pierce Co., Rockford, IL 61105, U.S.A.).

## 2) Substrate specificity

[0012] The substrate specificity of the enzyme was determined using the same enzyme assay method as described under 1) above, except that various substrate solutions ( 100 mM ) were used instead of L-sorbose. The relative activity of the ADH for D-glucosone, D-glucose, D-galactose, D-mannose, L-gulose, D-xylose, D-ribose and D-arabinose was higher than that for L-sorbose. However, the relative activity for D,L-glyceraldehyde was lower than 1 % of that for L-sorbose. These results are presented in Table 1:

Table 1

Substrate specificity of the purified enzyme	
Substrate	Relative activity (%)
L-Sorbose	100.0
D,L-Glyceraldehyde	<1
D-Glucosone	776.2
D-Glucose	864.2
L-Sorbose	<1
D-Galactose	949.1
D-Mannose	1003.3
L-Gulose	684.5
D-Sorbitol	<1
D-Xylose	1259.7
D-Ribose	803.9
D-Arabinose	298.9

## 3) Optimum pH

[0013] The correlation between the reaction rate of the ADH and pH values of the reaction mixture was determined by the same assay method as described under 1) above, except that various pHs and buffers were used.

[0014] The enzyme showed relatively high activity at pH 7.0 to 8.5, as shown in Fig. 1.

## 4) Thermostability

[0015] The thermostability of the enzyme was tested by incubating it for 5 minutes at various temperatures in 50 mM potassium phosphate buffer ( pH 7.0 ). The residual activity was measured by the same enzyme assay method as described under 1) above, after which the treated enzyme was immediately cooled down in ice water. The enzyme was

stable up to 45°C, but only about 30% of the activity remained after the treatment at 80°C. The results are shown in Table 2:

Table 2

Effect of temperature on the stability of the purified enzyme	
Temperature (°C)	Relative activity (%)
0	100.0
25	89.2
35	98.8
45	100.0
55	44.2
60	63.6
65	62.8
70	47.9
75	48.8
80	28.7

[0016] In this table the relative activities are expressed as percentages of the activity at 0°C.

#### 5) Effects of metal ions and inhibitors

[0017] The effects of metal ions and inhibitors on the activity of the enzyme were examined by measuring the activity using the same assay method as described under 1) above. Each compound solution was stirred into the basal reaction mixture and the reaction was started with the addition of the enzyme. The results are shown in Table 3:

Table 3

Effect of inhibitors and metals on the activity of the purified enzyme	
Compound	Relative activity (%)
None	100.0
EDTA	14.6
Quinine	124.4
KCN	129.4
NaN <sub>3</sub>	104.6
N-Ethylmaleimide	110.8
Monoiodoacetate	52.2
NaF	86.7
CaCl <sub>2</sub> · 2H <sub>2</sub> O	204.5
CoCl <sub>2</sub> · 6H <sub>2</sub> O	76.0
CuSO <sub>4</sub>	0.0
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> · xH <sub>2</sub> O	58.9
NiSO <sub>4</sub> · 6H <sub>2</sub> O	74.9

Table 3 (continued)

Effect of inhibitors and metals on the activity of the purified enzyme	
Compound	Relative activity (%)
TiCl <sub>4</sub>	128.0
ZnCl <sub>2</sub>	40.3
MgCl <sub>2</sub>	90.3

[0018] Each compound was added to the reaction mixture at a concentration of 1.0mM, except that the concentration of EDTA was 5.0 mM.

[0019] As shown in Table 3, the enzyme activity was stimulated by about 2-fold in the presence of 1.0 mM of Ca<sup>2+</sup>, whereas Co<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup> inhibited the enzyme activity. The addition of 5 mM ethylenediamine tetraacetic acid (EDTA) strongly inhibited the activity. However, the enzyme activity was slightly increased to 124% and 129% by the addition of 1.0 mM quinine and 1.0 mM KCN, respectively.

#### 6) Molecular weight

[0020] The molecular weight of the enzyme was measured with a size exclusion gel column (TSK-gel G3000 SWXL; Tosoh Co., Akasaka 1-7-7, Minato-ku, Tokyo, Japan). The enzyme showed two peaks corresponding to the apparent molecular weight of 150,000 ± 6,000 and 230,000 ± 9,000 on the chromatography. On analyzing this enzyme by SDS-polyacrylamide gel electrophoresis, it was shown that the enzyme consisted of the homologous subunit of molecular weight 75,000 ± 3,000 (Fig. 2). This indicates that the enzyme consists of two or three homologous subunits.

#### 7) Prosthetic group

[0021] The purified enzyme did not show the catalyzing activity for converting L-sorbose to 2-KGA in the basal reaction mixture without PQQ. However, the activity of the enzyme was restored by the addition of PQQ in the reaction mixture or incubating the enzyme with PQQ and Ca<sup>2+</sup> for 5 minutes.

[0022] The detection of heme c of the purified enzyme was performed by the reduced-minus-oxidized difference spectrum taken by a UV-VIS recording spectrophotometer (Shimadzu UV-2200; Shimadzu Co., Kuwahara-cho 1, Nishinokyo, Chukyo-ku, Kyoto, Japan). The enzyme was suspended in 50 mM potassium phosphate buffer (pH 7.0) at a concentration of 50 µg/ml and the enzyme of dithionite-reduced form and ammonium persulfate-oxidized form were prepared to measure the difference spectrum. The spectrum gave the difference maxima at 552 and 523 nm, as shown in Fig 3. The result strongly suggests that the enzyme has heme c as a prosthetic group.

#### 8) Effect of substrate concentration

[0023] The velocity of the oxidizing reaction with various concentrations of L-sorbose from 1 mM to 8 mM was measured to determine the *K<sub>m</sub>* value for L-sorbose. The Michaelis constant was calculated to be 17.8 mM from the Lineweaver-Burk plot based on the reaction velocity when DCIP was used as the electron acceptor for the reaction.

#### 9) Purification procedure

[0024] The purification of the enzyme is effected by any combination of known purification methods, such as ion exchange chromatography, gel-electrophoresis, salting out and dialysis.

[0025] The enzyme provided by the present invention can be prepared by cultivating an appropriate microorganism in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism.

[0026] The microorganisms used for the present invention are microorganisms belonging to the genus *Gluconobacter* which are capable of producing aldehyde dehydrogenase as defined hereinbefore. Functional equivalents, subcultures, mutants and variants of the said microorganism can also be used in the present invention.

[0027] A preferred strain is *Gluconobacter oxydans*. The strain most preferably used in the present invention is *Gluconobacter oxydans* DSM 4025, which was deposited at the Deutsche Sammlung von Mikroorganismen in Göttingen (Germany), based on the stipulations of the Budapest Treaty, under DSM No. 4025 on March 17, 1987. The depositor was The Oriental Scientific Instruments Import and Export Corporation for Institute of Microbiology, Academia Sinica,

52 San-Li-He Rd., Beijing, Peoples Republic of China. The effective depositor was said Institute, of which the full address is The Institute of Microbiology, Academy of Sciences of China, Haidian, Zhongguancun, Beijing 100080, People's Republic of China.

[0028] Moreover, a subculture of the strain has also been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, also based on the stipulations of the Budapest Treaty, under the deposit No. *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) on March 30, 1992. The depositor was the Nippon Roche Research Center, 200 Kajiwara Aza Sotokochi, Kamakura-shi, Kanagawa-ken 247, Japan. This subculture is also most preferably used in the present invention.

[0029] Furthermore, European Patent Publication No. 0 278 447 discloses the characteristics of this strain.

[0030] The microorganism may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at a pH of 4.0 to 9.0, preferably 6.0 to 8.0. The cultivation period varies depending on the pH, temperature and nutrient medium to be used, and is preferably about 1 to 5 days. The preferred temperature range for carrying out the cultivation is from about 13°C to about 36°C, preferably from 18°C to 33°C.

[0031] It is usually required that the culture medium contains such nutrients as assimilable carbon sources, for example glycerol, D-mannitol, D-sorbitol, erythritol, ribitol, xylitol, arabinol, inositol, dulcitol, D-ribose, D-fructose, D-glucose and sucrose, preferably D-sorbitol, D-mannitol and glycerol; and digestible nitrogen sources such as organic substances, for example, peptone, yeast extract, baker's yeast, urea, amino acids and corn steep liquor. Various inorganic substances may also be used as nitrogen sources, for example nitrates and ammonium salts. Furthermore, the culture medium usually contains inorganic salts, for example magnesium sulfate, potassium phosphate and calcium carbonate.

[0032] An embodiment for the isolation and purification of the ADH from the microorganism after the cultivation is briefly described hereinafter:

(1) Cells are harvested from the liquid culture broth by centrifugation or filtration.

(2) The harvested cells are washed with water, physiological saline or a buffer solution having an appropriate pH.

(3) The washed cells are suspended in the buffer solution and disrupted by means of a homogenizer, sonicator or French press or by treatment with lysozyme and the like to give a solution of disrupted cells.

(4) The ADH is isolated and purified from the cell-free extract of disrupted cells, preferably from the cytosol fraction of the microorganism.

[0033] The ADH provided by the present invention is useful as a catalyst for the production of 2-KGA from L-sorbose. The reaction should be conducted at pH values of about 6.5 to about 9.0 in the presence of an electron acceptor, for example DCIP, PMS and the like in a solvent such as phosphate buffer, Tris-buffer and the like. When the pH and temperature are set at about 7.5 to 8.5 and about 25°C, respectively, the reaction usually produces the best results.

[0034] The concentration of L-sorbose in a solvent can vary depending upon other reaction conditions but, in general, is about 0.5 to 50 g/l, most preferably from about 1 to about 30 g/l.

[0035] In the reaction, the ADH may also be used in an immobilized state with an appropriate carrier. Any means of immobilizing enzymes generally known in the art may be used. For instance, the enzyme may be bound directly to a membrane, granules or the like of a resin having one or more functional groups, or it may be bound to the resin through bridging compounds having one or more functional groups, for example glutaraldehyde.

[0036] In addition to the above, the cultured cells are also useful for the production of carboxylic acids from aldehydes, especially for the production of 2-KGA from L-sorbose.

[0037] The following Example further illustrates the present invention.

### Example

#### Preparation of ADH

[0038] All the operations were performed at 8°C, and the buffer was 0.05 M potassium phosphate (pH 7.0) unless otherwise stated.

(1) Cultivation of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812)

[0039] *Gluconobacter oxydans* DSM 4025 (FERM BP-3812) was grown on an agar plate containing 5.0% D-mannitol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.75% corn steep liquor, 5.0% baker's yeast, 0.5% urea, 0.5%  $\text{CaCO}_3$  and 2.0% agar at 27°C for 4 days. One loopful of the cells was inoculated into 50 ml of a seed culture medium containing 2% L-sorbose, 0.2%

yeast extract, 0.05% glycerol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.75% corn steep liquor, 0.5% urea and 1.5%  $\text{CaCO}_3$  in a 500 ml Erlenmeyer flask, and cultivated at 30°C with 180 rpm for one day on a rotary shaker. 10 ml samples of this culture were transferred into 500 ml Erlenmeyer flasks containing 100 ml of the same seed culture medium and cultivated in the same manner as described above. The seed culture thus prepared was used for inoculating 15 liters of medium, which contained 8.0% L-sorbose, 0.05% glycerol, 0.25%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3.0% corn steep liquor, 0.4% yeast extract and 0.15% antifoam, in 30 l jar fermentor. The fermentation parameters were 800 rpm for the agitation speed and 0.5 vvm (volume of air / volume of medium / minute) for aeration at a temperature of 30°C. The pH was maintained at 7.0 with sodium hydroxide during the fermentation. After 48 hours of cultivation, 30 liters of the cultivated broth containing the cells of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) by using the two sets of fermentors were harvested by continuous centrifugation. The pellets containing the cells were recovered and suspended in an appropriate volume of saline. After the suspension had been centrifuged at 2,500 rpm (1,000 x g), the supernatant containing the slightly reddish cells was recovered to remove the insoluble materials derived from corn steep liquor and yeast extract which were ingredients in the medium. The supernatant was then centrifuged at 8,000 rpm (10,000 x g) to obtain the cell pellet. As a result, 123 g of the wet weight of cells of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812) were obtained from 30 liters of broth.

## (2) Preparation of cytosol fraction

[0040] The cell paste (55 g) was suspended with 100 ml of the buffer and passed through a French pressure cell press. After centrifugation to remove intact cells, the supernatant was designated as the cell-free extract, and the cell-free extract was centrifuged at 100,000 x g for 90 minutes. The resultant supernatant (165 ml) was designated as the soluble fraction of *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812). After this fraction had been dialyzed against the buffer, 126 ml of the dialyzed fraction having the specific activity on L-sorbose of 2.26 units/mg protein were used for the next purification step.

## (3) Diethylaminoethyl (DEAE)-cellulose column chromatography

[0041] The dialysate (126 ml) was put on a column of DEAE-cellulose (Whatman DE-52, 3 x 50 cm; Whatmann Bio-Systems Ltd., Springfield Mill, James Whatman Way, Maidstone, Kent, U.K.) equilibrated with the buffer and washed with the buffer to elute minor proteins. Then a linear gradient elution with NaCl from 0.3 to 0.8 M in the buffer was carried out. Major enzyme activity was eluted at NaCl concentrations ranging from 0.32 to 0.36 M. The active fractions (116 ml) were collected and dialyzed against the buffer.

## (4) DEAE-sepharose column chromatography

[0042] A 60 ml portion of the dialyzed active fraction from the previous step was introduced into a column of DEAE-sepharose CL-6B (Pharmacia, 1.5 x 50 cm; Amersham Pharmacia Biotech AB, S-75184 Uppsala, Sweden) equilibrated with the buffer. After the column had been washed with the buffer containing 0.2 M NaCl, a linear gradient of NaCl from 0.2 to 0.6 M was added to the buffer. The active fractions were eluted at NaCl concentrations ranging from 0.44 to 0.47 M.

## (5) Q-sepharose column chromatography

[0043] A portion (13.5 ml) of the pooled active fractions (53 ml) from the previous step was added with appropriate volume of the buffer to decrease the concentration of NaCl, and introduced into a column of Q-sepharose (Pharmacia, 1.0 by 20 cm) equilibrated the buffer. After the column had been washed with the buffer containing 0.35 M NaCl, a linear gradient of NaCl from 0.35 to 0.5 M was added to the buffer. The activities corresponding to the ADH were eluted at NaCl concentrations ranging from 0.39 to 0.40 M. The active fractions (30 ml) collected were ultrafiltrated by an ultrafiltrator (Centriprep-10, Amicon; Amicon Inc. Cherry Hill Drive, Beverly, MA 01915, U.S.A.) to concentrate and desalt. As a result, 700 µl of the concentrated active fraction were obtained.

## (6) Native polyacrylamide gel electrophoresis (Native PAGE)

[0044] A 600 µl portion of the enzyme fraction from the previous step was applied on a native polyacrylamide gel (10%, pH 9.4, 10 by 10 cm). The electrophoresis was performed at 30 mA and 4°C for 1.5 hours. The enzyme band corresponding to the active fraction was excised from the gel, and the enzyme was electrically eluted from the gel into the Tris glycine buffer (pH 8.3) by using a MAX-YIELD Protein Concentrator (Atto Co., Hongo 1-25-23, Bunkyo-ku, Tokyo, Japan) at 10 W and 4°C for 3 hours. The enzyme solution was concentrated 4-fold using an ultramembrane filter (Cen-

tricon-10, Amicon), and the buffer was changed to 50 mM potassium phosphate buffer ( pH 7.0 ). Then the enzyme solution was stored at -30°C.

[0045] A summary of the purification steps of the enzyme is given in Table 4.

Table 4

Purification of the aldehyde dehydrogenase from <i>Gluconobacter oxydans</i> DSM No.4025 (FERM BP-3812)			
Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)
Soluble fraction	5994.2	2652.3	2.26
DEAE-Cellulose DE52	4206.9	594.2	7.08
DEAE-Sepharose CL-6B	1640.5	107.9	15.29
Q-Sepharose	243.3	11.84	20.55
Native PAGE	193.8	3.59	53.98

#### (7) Purity of the isolated enzyme

[0046] The purified enzyme with a specific activity of 54.0 units per mg protein (0.62 mg/ml ) was used for the following analysis:

[0047] The molecular weight of the native enzyme was determined by high performance liquid chromatography using a size exclusion gel column (TSK gel G3000 SWXL column, 7.8 x 300 mm ) equilibrated with 0.1 M potassium phosphate buffer ( pH 7.0 ) containing 0.3 M NaCl at 280 nm and a flow rate of 1.5 ml per minute. Cyanocobalamin ( 1.35 K ), myoglobin ( 17 K ), ovalbumin ( 44 K ),  $\gamma$ -globulin ( 158 K ) and thyroglobulin ( 670 K ) were used as molecular weight standards. The purified enzyme showed two peaks having the molecular weights  $150,000 \pm 6,000$  and  $230,000 \pm 9,000$ .

[0048] However, in the presence of sodium dodecyl sulfate ( SDS ), the enzyme showed a single band with a molecular weight of  $75,000 \pm 3,000$ . From these results, the purified enzyme consisted of two or three homologous subunits.

#### (8) Identification of the reaction product

[0049] The reaction mixture containing the purified enzyme ( 1.56 mg ), L-sorbose ( 0.142 mg ), PMS ( 0.008 mg ) and PQQ ( 0.3 mg ) in 40 ml of the buffer was incubated for 1.5 hours at 30°C. The reaction product was analyzed on thin layer chromatography and HPLC. As a result, the reaction product was identified as 2-KGA in comparison with an authentic sample of 2-KGA.

#### Claims

1. An aldehyde dehydrogenase having the following physico-chemical properties :

a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$  )

b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone and heme c

d) Optimum pH : 7.0 to 8.5

e) Inhibitors :  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid.

2. The aldehyde dehydrogenase according to claim 1, which is derived from a microorganism belonging to the genus *Gluconobacter* which is capable of producing the said enzyme having the physico-chemical properties as given in claim 1.

3. The aldehyde dehydrogenase according to claim 2, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812).

4. The aldehyde dehydrogenase according to claim 3, wherein the microorganism corresponds to *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

5. A process for producing the aldehyde dehydrogenase having the following physico-chemical properties:

a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$ )

b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone and heme c

d) Optimum pH : 7.0 to 8.5

e) Inhibitors :  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid

which comprises cultivating a microorganism belonging to the genus *Gluconobacter*, which is capable of producing the aldehyde dehydrogenase having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism.

6. The process according to claim 5, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812).

7. The process according to claim 6, wherein the microorganism corresponds to *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

8. A process for producing 2-keto-L-gulonic acid from L-sorbose which comprises contacting L-sorbose with an aldehyde dehydrogenase having the following physico-chemical properties:

a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$ )

b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone and heme c

d) Optimum pH : 7.0 to 8.5

e) Inhibitors:  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid

in the presence of an electron acceptor, and isolating the resulting 2-keto-L-gulonic acid from the reaction mixture.

9. A process for producing 2-keto-L-gulonic acid from L-sorbose which comprises contacting L-sorbose with a microorganism belonging to the genus *Gluconobacter* which is capable of producing the aldehyde dehydrogenase having the following physico-chemical properties:

a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$ )

b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone and heme c

d) Optimum pH : 7.0 to 8.5

e) Inhibitors :  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid

in an aqueous nutrient medium under aerobic conditions, and isolating the resulting 2-keto-L-gulonic acid from the reaction mixture.

5 10. The process according to claim 9, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812).

10 11. The process according to claim 10, wherein the microorganism corresponds to *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

12. A process for producing 2-keto-L-gulonic acid from L-sorbose which comprises contacting L-sorbose with a cell-free extract of a microorganism belonging to the genus *Gluconobacter* which is capable of producing the aldehyde dehydrogenase having the following physico-chemical properties:

15 a) Molecular weight :  $150,000 \pm 6,000$  or  $230,000 \pm 9,000$  (consisting of two or three homologous subunits, each having a molecular weight of about  $75,000 \pm 3,000$  )

20 b) Substrate specificity : active on aldehyde compounds

c) Cofactors : pyrroloquinoline quinone and heme c

d) Optimum pH : 7.0 to 8.5

25 e) Inhibitors :  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , monoiodoacetate and ethylenediamine tetraacetic acid

and isolating the resulting 2-keto-L-gulonic acid from the reaction mixture.

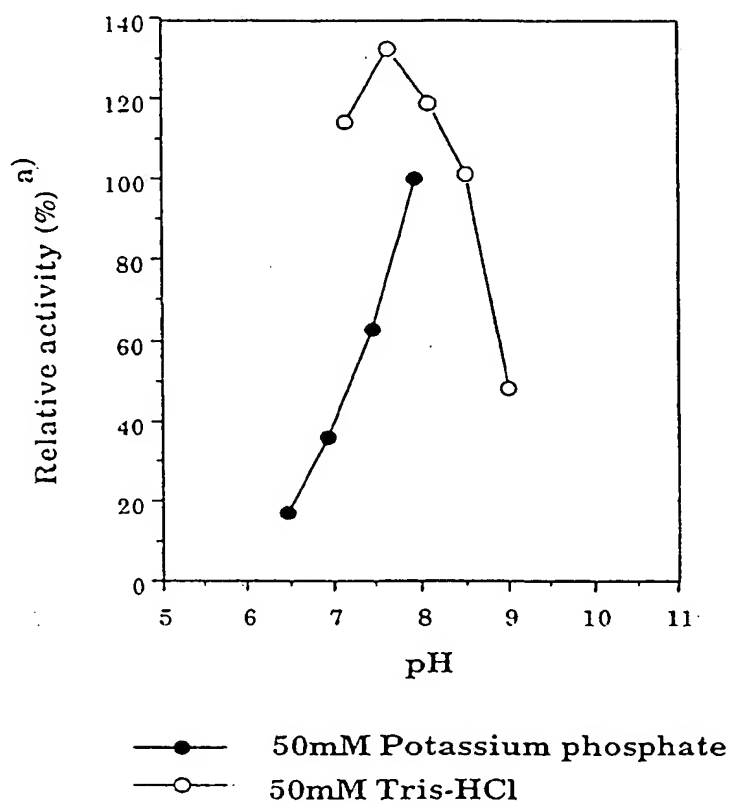
30 13. The process according to claim 9, wherein the microorganism is *Gluconobacter oxydans* having the identifying characteristics of the strain *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812).

35 14. The process according to claim 10, wherein the microorganism corresponds to *Gluconobacter oxydans* DSM No. 4025 (FERM BP-3812), a functional equivalent, subculture, mutant or variant thereof.

Fig. 1

### Effect of pH on the activity of the purified enzyme

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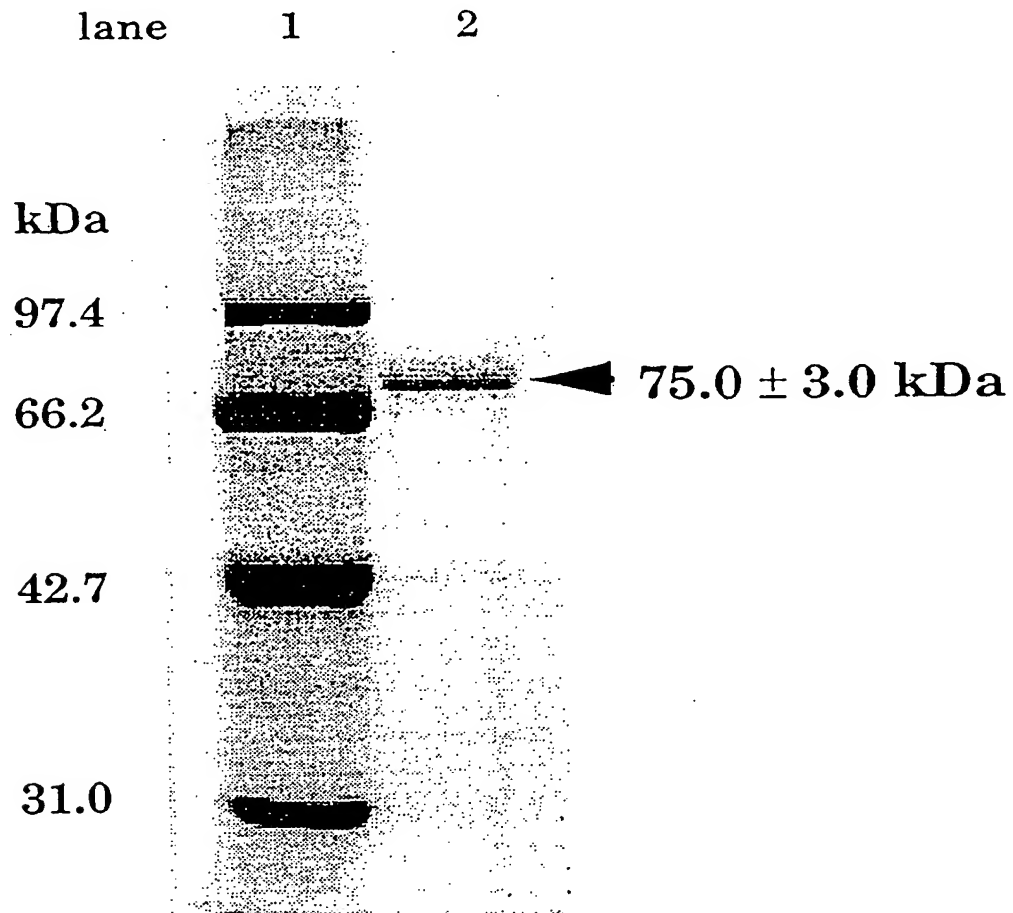


a) Data are expressed as a percentage of the activity at pH 8.0 of potassium phosphate buffer.

Fig. 2

SDS-PAGE analysis of the purified enzyme

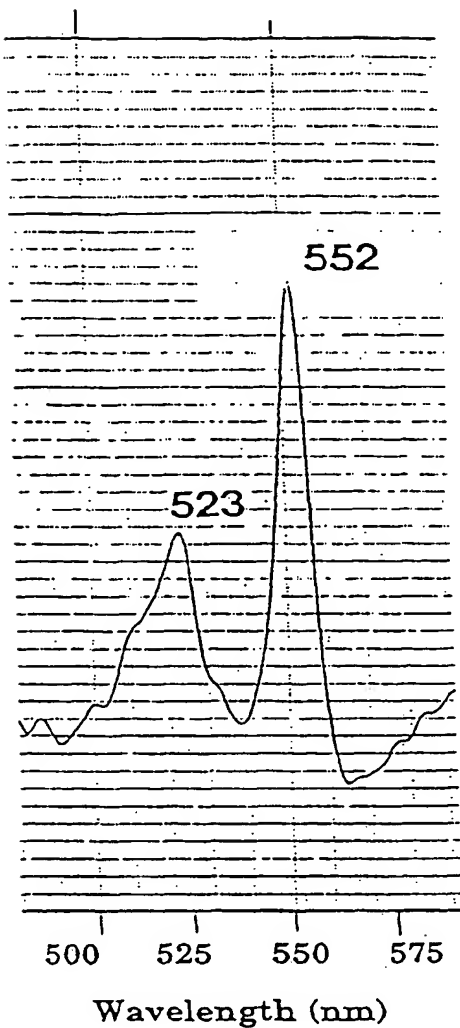
10% gel  
CBB staining



lane 1: Molecular weight standards: phospholylase B, 97.4k; bovine serum albumin, 66.2k; ovalbumin, 42.7k; bovine carbonic anhydrase, 31.0k.

lane 2: The purified enzyme treated with SDS.

Fig. 3



Reduced minus oxidized difference spectrum of the purified enzyme protein.

(19)



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(11)

**EP 0 922 759 A3**

(12)

**EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:  
19.01.2000 Bulletin 2000/03

(51) Int. Cl.<sup>7</sup>: **C12N 9/02, C12P 7/60**  
// (C12N9/02, C12R1:01)

(43) Date of publication A2:  
16.06.1999 Bulletin 1999/24

(21) Application number: 98122223.5

(22) Date of filing: 23.11.1998

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU**  
**MC NL PT SE**  
Designated Extension States:  
**AL LT LV MK RO SI**

(30) Priority: 01.12.1997 EP 97121089

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(54) **Aldehyde dehydrogenase**

(57) A new aldehyde dehydrogenase having the physico-chemical properties: molecular weight: 150,000 ± 6,000 or 230,000 ± 9,000; substrate specificity: active on aldehyde compounds; cofactors: pyrrolo-quinoline quinone and heme *c*; optimum pH: 7.0-8.5; and inhibitors: Co<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, monoiodoacetate and EDTA, is derived from a microorganism belonging to the genus *Gluconobacter*. Said aldehyde dehydrogenase can be produced by cultivating a microorganism of the genus *Gluconobacter* which is capable of producing an aldehyde dehydrogenase having the above properties, in an aqueous nutrient medium under aerobic conditions, disrupting the cells of the microorganism and isolating and purifying the aldehyde dehydrogenase from the cell-free extract of the disrupted cells of the microorganism. 2-Keto-L-gulonic acid (2-KGA) can be produced from L-sorbose by contacting L-sorbose with (i) the aldehyde dehydrogenase in the presence of an electron acceptor, (ii) a *Gluconobacter* microorganism capable of producing the aldehyde dehydrogenase in an aqueous medium under aerobic conditions or (iii) a cell-free extract of said microorganism, and in each case isolating the resulting 2-KGA from the reaction mixture.

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# EUROPEAN SEARCH REPORT

Application Number  
EP 98 12 2223

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	EP 0 606 621 A (HOFFMANN LA ROCHE) 20 July 1994 (1994-07-20) * page 3, line 35-50 * * table 1 * * page 4, line 14-24 * * table 2 * * table 6 * * table 7 * * table 9 * * claims 1-11 *	1-14	C12N9/02 C12P7/60 /(C12N9/02, C12R1:01)
Y	MA-SUSHITA K ET AL: "Function of multiple heme c moieties in intramolecular electron transport and ubiquinone reduction in the quinohemoprotein alcohol dehydrogenase-cytochrome c complex of Gluconobacter suboxydans." JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 MAR 1) 271 (9) 4850-7. , XP002122097 * table II * * page 4853, paragraph 3 *	1-14	
A	THURNER, CLAUDIA ET AL: "Biochemical and genetic characterization of the acetaldehyde dehydrogenase complex from Acetobacter europaeus" ARCH. MICROBIOL., vol. 168, no. 2, 1997, pages 81-91, XP000852981 * figure 1C * * page 90, paragraph 4 *	1-14	C12N C12P
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 17 November 1999	Examiner ALCONADA RODRIG., A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.82 (P04C01)



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# EUROPEAN SEARCH REPORT

Application Number  
EP 98 12 2223

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	KONDO K ET AL: "Characterization of the genes encoding the three-component membrane-bound alcohol dehydrogenase from gluconobacter suboxydans and their expression in Acetobacter pasteurianus" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 3, March 1994 (1994-03), pages 1131-1138, XP002914612 ISSN: 0099-2240 * page 1134 *	1-14	
A	EP 0 790 301 A (HOFFMANN LA ROCHE) 20 August 1997 (1997-08-20) * example 2 * * claims 8-10 *	5-14	
A	EP 0 758 679 A (FUJISAWA PHARMACEUTICAL CO) 19 February 1997 (1997-02-19) * the whole document *	1-14	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 17 November 1999	Examiner ALCONADA RODRIG., A
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EPO FORM 1503 03.82 (P/C01)

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ON EUROPEAN PATENT APPLICATION NO.**

EP 98 12 2223

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17-11-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0606621 A	20-07-1994	JP 7000182 A	06-01-1995
		US 5437989 A	01-08-1995
		US 5932463 A	03-08-1999
		US 5916785 A	29-06-1999
EP 0790301 A	20-08-1997	CN 1171441 A	28-01-1998
		JP 9224660 A	02-09-1997
		US 5776742 A	07-07-1998
EP 0758679 A	19-02-1997	US 5834263 A	10-11-1998
		US 5888786 A	30-03-1999
		CN 1146780 A	02-04-1997
		WO 9523220 A	31-08-1995

EPO FORM P449

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